ANTITUMOUR VECTOR CONSTRUCTS AND METHODS

The present invention relates to the control of proliferation of tumour cells, preferably by killing those cells. More particularly it relates to methods
5 and means for selectively attacking tumour cells with cytotoxic or antiproliferative agents (herein generally referred to as antitumour agents).

The concept of targeting antitumour agents to tumour cells has been considered and investigated over
10 a number of years. Such approaches include· for example the use of supposedly tumour-specific antibodies or ligands as carriers for cytotoxic agents, but this has problems, such as finding antigens or ligand receptors which occur on all tumour
15 cells and virtually not at all on normal cells; also the problem of physically getting the conjugate to the target tumour cells.

A variety of gene therapy strategies have been proposed, some of which are currently in clinical
20 trials. These include *ex-vivo* introduction of cytokines into explanted tumour cells or into tumour infiltrating lymphocytes (TIL) followed by re-introduction of these cells into patients, in the hope of stimulating tumour recognition and elimination by
25 the immune system [1].

A complementary approach has been to introduce pro-drug activating enzymes, the most common of which is the herpes simplex virus thymidine kinase gene

(HSVTK) which can metabolise the pro-drug, Ganciclovir, to a cytotoxic metabolite capable of killing the host cell [2]. A major advantage of this strategy is that the so called "bystander effect"

5   limits the requirement to target the gene to all of the tumour cells. Using the TK gene, the cytotoxic drug which is generated can pass through gap junctional channels into surrounding cells [3], with the consequence that targeting of only 10-20% of the

10   cells within a tumour can lead to complete tumour regression. For all proposed new cancer therapies, a similar series of problems have to be resolved, including the means of delivery of any new drug, optimisation of its uptake and stability, specificity

15   for targeting of tumour versus normal cells and the necessity to ensure that all tumour cells are exposed to the drug.

The development of gene therapy faces exactly the same difficulties, but probably the most complicated

20   challenge to be overcome is the ability to engineer tumour-specific expression of the targeted gene. The most commonly used method to try and circumvent this problem is to use a tissue-specific gene promoter to drive expression of the therapeutic gene in particular

25   tumour types. For example, the tyrosinase promoter is currently being used to express TK in melanoma cells [4]. However, this promoter is active in normal melanocytes as well as in some neurones. An

3

alternative is to utilise the promoters of genes which are upregulated in tumours such as the erbB2 gene in breast cancers [5]. This gene is, however, also expressed in normal cells and in any case its

5 deregulation only occurs in a relatively small proportion (20-30%) of human breast, pancreatic and gastric tumours. All of the presently available systems therefore suffer from the disadvantages that specificity for normal versus tumour cells is weak and

10 the applicability is restricted to very specific tumour types.

One objective of the present invention, therefore, is to obtain improved discrimination in the action of antitumour agents between tumour and normal

15 cells.

In broad terms, selectivity for expression within tumour cells of the anti-tumour agent in accordance with the present invention will be effected by a combination of two approaches: (i) up-regulation of

20 the mediating gene in tumour cells and (ii) down-regulation of the mediating gene in normal cells. Effect (i) will mediate the desired activity in and around tumour cells, while effect (ii) will reduce the extent of "leaky" expression of that activity in

25 normal cells.

A preferred basis for this approach is centred around the p53 gene. The p53 protein is a transcriptional activator in normal cells but is

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4

present in mutant form in a substantial proportion
(40-80%) of human tumours. However, mutation
frequencies of p53 is likely to underestimate the
actual occurence of inactivation of the p53 tumour
5   suppressor function in tumours. Even in tumours in
which the p53 sequence is wild-type, its normal
function in cell cycle control, DNA repair,
differentiation, genome plasticity or apoptosis [43-
45] may be abrogated, for instance by interaction with
10 cellular protein (e.g. mdm2) or oncoviral protein
(e.g. SV40 T antigen, human papillomavirus E6 protein,
adenovirus E1B protein, hepatitis B virus X protein,
and Epstein-Barr BZLF-1 protein), or by being
sequestered in the cytoplasm, where the p53 protein is
15 non-functional.

    The strategy exploits differences in the
transcriptional activation and stability functions of
the p53 protein between normal and tumour cells. More
particularly, it utilises the dual transcriptional
20 regulatory function of wild-type p53 (wtp53) to
eliminate or minimise expression of the antitumour
gene in normal cells and to enhance its expression in
tumour cells. For this purpose, the strategy proposes
a combination of two genetic units. In genetic unit
25 1, the antitumour gene is controlled by a promoter
whose function is suppressed by wtp53, but is not
suppressed by mutant p53 (mp53), indeed it may even be
up-regulated by mp53. In genetic unit 2, a gene for

down-regulating the antitumour gene in normal cells is controlled by a promoter containing the p53 binding site, and which is therefore potently up-regulated by wtp53 but not by mp53. In other words, the wtp53 in normal cells is used to up-regulate a gene which down-regulates the expression of the antitumour agent in those normal cells. The down-regulating gene may for example express antisense RNA to the antitumour gene, or a specific ribozyme for antitumour gene RNA, or a specific transcription suppressor for the antitumour gene.

By way of example, this system can be used to engineer expression in tumour cells of a pro-drug activating enzyme such as HSVTK, but it will be useful for targeting of genes which enhance host anti-tumour response such as IL-2 and GMCSF, cytokines, genes which may induce tumour specific apoptosis, such as bax, or other toxins or functions such as viral replication capacity, e.g. viruses capable of replication only in tumour cells, to tumour cells.

Exemplary embodiments of dual genetic unit constructs according to the present invention are illustrated diagrammatically in Figures 1A and 1B, with reference to expression of HSVTK as the antitumour gene and p53-based regulation, and will be described further below.

The invention is preferably based on the p53 gene, because of its central role in tumour

suppression and the differences in its function in normal and tumour cells. However, the general concept of the invention may be applicable using other genes to up-regulate the antitumour agent in tumour cells

5   and to down-regulate it in normal cells. Since the natural tumour suppressor pathways are complex and involve many genes, at least some of them could be used in a similar fashion.

10   *More detailed discussion of the invention in relation to p53*

The most common feature of virtually all malignant tumour cells is aneuploidy. There is a great deal of evidence showing that tumour cells are

15   genetically unstable, possibly because of the accumulation of mutations in genes which control the stability and integrity of the genome, such as p53 [6]. A consequence of such genetic changes is that tumour cells may constitutively produce signals

20   indicating DNA damage. The main aim of the p53 aspect of this invention to engineer gene constructs which will utilise the intrinsic "DNA damage" response in tumour cells to switch.on and stabilise an antitumour agent, such as a pro-drug activating enzyme.

25   We envisage a number of avenues by which tumour specific targetting of gene expression of an antitumour agent may be achieved. Central to the presently preferred proposal are the observations that

the p53 tumour suppressor gene can act as a sensor of
DNA damage or aneuploidy in developing tumour cells
[6]. In normal cells, p53 responds to DNA damage
induced by exogenous agents initially by increasing

5   the stability of the protein itself and/or by
increasing the translatability of its mRNA [7].
Importantly, the introduction of the same mutant form
of p53 into tumour cell lines showing different
expression levels of endogenous p53 genes has shown

10   that the exogenous p53 is stabilised in the same way
as its endogenous counterpart [8]. In other words,
the level of protein stability and expression is not
simply a consequence of mutation within the coding
sequence but is determined by the tumour cell

15   microenvironment.

Stabilisation of p53 in normal cells can lead to
apoptosis directly or can cause G1 arrest through the
induction of genes such as p21/Waf-1 [9]. In many
tumour cells these responses are abrogated, either

20   because of deletion of p53 wild type sequences or by
mutation to other conformational forms which fail to
induce these responses. Interestingly, although the
majority of these mutants fail to activate
transcription through the recognition sequence

25   discovered in genes such as p21/Waf-1, some of them
can nevertheless stimulate transcription from some
other gene promoters which are known to be upregulated
in certain tumour cells, such as the MDR1 gene [10] or

8

Hsp70 gene [11] promoters.

Since it appears extremely unlikely that the use
of a single tissue-specific or tumour-selective gene
promoter to drive eg TK expression will give
5   sufficient discrimination between normal and tumour-
cells, the present invention proposes the use of both
altered transcriptional control by wild-type and
mutant p53 and altered translation or stability of the
p53 protein itself to control the expression of eg
10   HSVTK for the purpose of gene therapy. Such
transcriptional control and such translational or
stability control may be used individually or in
combination. The constructs are tested initially *in
vitro*, but will ultimately be rigorously tested for
15   tumour specificity *in vivo* by using human tumour
xenografts in nude mice and transgenic mice.

For convenience, initial studies are carried out
with marker genes such as luciferase, as well as with
HSVTK (pro-drug activating enzyme), but these will
20   then be extended to other systems. The advantages of
the proposed system include wide applicability to a
range of tumour cell types and the ability to extend
the system to include tumour specific expression of
other potentially therapeutic genes in addition to
25   pro-drug activating enzymes, eg genes which enhance
host anti-tumour response, such as IL-2, GMCSF,
cytokines, genes which may induce tumour specific
apoptosis, such as bax, toxins or genes which control

9

viral replication capacity, for instance viruses
capable of replicating only in tumour cells.

p53 mediated transcriptional control

Wild-type p53 protein contains at least 4
functional domains, one of which (residues 102-290)
binds to two copies of the consensus sequence 5'-
PuPuPuC(A/T)(A/T)GpyPyPy-3' [12-15]. GCCC + a dimer
of the consensus sequence has been used herein, as
discussed below, and is known as GC_3p53 (the sequence
being exemplified using $GC_3(GGACTTGCCT)_2$).
Transactivation through this sequence increases the
level of transcription of a number of cellular genes,
some of which play major roles in the negative
regulation of cell proliferation or in triggering
programmed cell death (apoptosis).

A p53 consensus sequence is present within the 5'
region of the gene encoding the p21/Waf-1 cdk
inhibitor, which can arrest the cell cycle in the late
G1 phase [16] and inhibits the function of
proliferating cell nuclear antigen (PCNA), a
regulatory subunit of DNA polymerase delta [17].

Up-regulation of the gene encoding the Bax
protein, which promotes apoptosis by inhibition of
Bcl-2, has also been reported to be induced by wild-
type p53 [18].

This form of transactivation appears to be
limited to promoters containing the p53 consensus

binding site, whereas the promoters of a number of
other cellular genes including Bcl-2 [19], PCNA [20,
21], the multi-drug resistance gene MDR1 [10] and the
HSP70 genes [11] are negatively regulated by wild-type

5   p53. The latter effect probably involves complex
formation of wild-type p53 with various components of
the basal transcription machinery [22-25].

Most of the mutations in the p53 gene lead to
abrogation of the sequence-specific transcriptional ·

10   activating function and prevent the suppression of
tumour cell growth in vitro. This has been attributed
to dominant negative suppression of wild-type p53
function in heterozygous cells, but in some cases gain
of function properties have been ascribed to some

15   mutant forms [26]. This gain of function could be due
to the reported ability of some mutant p53 alleles to
transactivate promoters which are normally suppressed
by wild-type p53 [11]. For example, both PCNA and
MDR1 promoters, in addition to the HSP70 promoter,

20   have been reported to be transactivated by several
mutant p53 genes [20, 27].

Gene promoters which exhibit these opposite
transcriptional responses to wild-type and mutant p53
may be extremely useful for tumour specific gene

25   targeting. A construct driven by either the MDR1 or
HSP70 promoters would be expected to be expressed at
high levels in tumour cells carrying mutant p53 genes
but suppressed in normal cells, expressing wild-type

11

p53. However, although there may be some selective activity in the use of these promoters, complete discrimination between normal and tumour cells would not be expected. We therefore incorporate an

5 additional control element designed to suppress expression specifically in normal cells (as illustrated by way of example in Fig 1). The HSP70 promoter is a good choice of promoter for the Type I unit because its overall strength is relatively

10 moderate, which makes it more likely that complete suppression can be achieved in normal cells with wild-type p53.

The initial constructs comprise two separate genetic units. The HSVTK gene is driven by a promoter

15 (type I promoter) which is not suppressed by, and may even be stimulated by, mutant p53 but is suppressed by wild-type p53 (eg the HSP70, MDR1 or PCNA promoters). The second genetic unit is designed to suppress any leaky expression of the TK gene in normal cells. The

20 promoter for this unit (type II promoter) contains the p53 consensus binding sequence, which is only activated by wild-type p53, within the context of a minimal promoter comprising a transcriptional start site and TATA Box. The p53 binding sequence may be

25 included upstream of the minimal promoter or downstream. Experimental results included herein show that in one exemplary system better results are achieved with the p53 binding sequence downstream from

the promoter in genetic unit II.

Apart from p53, other genes associated with the tumour suppression pathway may be utilised in the present invention. For example, the p16 INK4A gene has a tumour suppressor function. It inhibits phosphorylation of Rb protein, which in turn negatively regulates the p16 promoter. Many tumours have Rb or p16 deletions, but not both, so that Rb-negative tumours tend to have high p16 levels. The p16 promoter should therefore be strongly up-regulated in Rb-negative tumours, and could be used as the type I promoter in our dual construct.

Various means of suppressing leaky transcription in normal cells may be employed and three different approaches have been considered experimentally (results provided below):

(i) One approach employs an antisense construct (e.g. for HSVTK) driven by the type II promoter (e.g. p53 responsive). No antisense transcript should therefore be produced in tumours harbouring only mutant p53, but this construct should be transcriptionally activated within normal cells and lead to down-regulation of any functional TK transcript driven by the type I promoter. It should be noted that even although normal cells express only very low levels of wild-type p53, the introduction of gene constructs by transfection has been shown to induce expression of detectable wild-type p53 [28].

Such induction would therefore help to improve
discrimination between normal and tumour cells using
the construct shown in Fig 1. Low dose radiation,
which would be expected to induce wt p53 in normal but
5   not tumour cells, may also improve discrimination.

(ii) Another approach to suppressing the level of
activity of a polypeptide such as TK in normal cells
involves the use of a specific ribozyme [29] designed
to complex with and cleave the target (e.g. TK) mRNA.

10   (iii) A third approach uses a sequence-specific
transcriptional suppressor. Several such
transcriptional suppressors have been characterised.
Experiments may include use of the binding domain of
the yeast Gal-4 transcription factor and the
15   suppression domain of the Drosophila even-skipped
protein [30] under the control of the type II promoter
to suppress expression by means of a tandem repeat of
the Gal-4 target sequence [31] cloned into the type I
promoter. Further experiments are described below,
20   e.g. involving lacI and tet repressor systems.

A combination of two or more such approaches may
be utilised. However, the most appropriate approach
may vary according to the antitumour gene being
expressed.

25   If antisense or ribozyme technology is employed,
the sequence expressed by the Type II unit must be
tailored to the sequence expressed by the Type I unit,
adding a level of complexity to experimentation. In

contrast, the use of a transcriptional suppressor has
the advantage of allowing the construction of a
"universal cassette" into which different coding
sequences, e.g. anti-tumour or marker genes, may be
5   inserted under the control of the Type I promoter
without there being a need for tailoring of the Type
II unit.

In experimental optimisation of the antitumour
system of the present invention, initially the type I
10   and type II promoter constructs may be tested
individually, to assess the magnitude of the effects
which can be generated. Co-transfections may then be
carried out to assess the combined effects of both
constructs in determining overall expression levels of
15   a reporter gene such as luciferase in the presence of
wild-type or mutant p53. Finally, both genetic units
may be incorporated into a single construct in either
a head-to-head (as in Fig 1), tail-to-tail or head-to-
tail arrangement, in the latter case having the type
20   II promoter upstream of the type I promoter, or vice
versa. If it is observed that there is
transcriptional or enhancer interference between the
two constructs, this may be overcome by altering the
relative distance between and/or orientation of the
25   units, and/or by linearisation of the construct,
and/or (see Fig 1) by the introduction of so-called
interruptor (insulator) sequences, which have been
characterised for example in Drosophila [32,33] and

chicken [34,42] systems, and shown to prevent
interactions between enhancer elements in adjacent
transcription units.

5   *p53-mediated stabilisation*

An aspect of the present invention provides a
composition wherein a first gene of which the
expression product is desired to be stabilised in
tumour cells is provided as a fusion with a sequence
10   encoding p53 protein or a fragment of p53 protein
which is stabilised in tumour cells. Thus, a nucleic
acid construct may include a gene encoding a
polypeptide which is a fusion of a polypeptide of
interest and p53 protein or a fragment of p53 protein
15   which is stabilised in tumour cells.

Experimental evidence provided herein shows that
stability of such fusion proteins is regulated in the
same manner as wild-type p53. The fusions are
stabilised in cells under conditions in which wild-
20   type p53 is stabilised. Such constructs may be useful
for targetting functional expression of a polypeptide,
such as an antitumour agent, to tumour cells which
either express a mutant p53 or express wild-type p53
but with abrogated function, e.g. because of abnormal
25   location within the cell (such as in the cytoplasm -
as discussed elsewhere herein).

The use of p53-mediated stabilisation in
targetting functional expression within tumour cells

as opposed to non-tumour cells may be combined with
the transcriptional control approach discussed above
and below.

5   Compositions, cells and methods according to the
present invention may be used in methods in which
expression of a desired gene is targetted to tumour
cells, as opposed to non-tumour cells. Such methods
may be performed *in vivo* (e.g. by way of treatment of
10   a human or animal body for therapeutic purposes), *ex
vivo* (e.g. on cells removed from a human or animal
body, prior to return of the cells to the body) or *in
vitro*. Compositions and cells may be used in the
manufacture of a medicament for treatment in which
15   expression of a desired gene is targetted to tumour
cells. Nucleic acid constructs may form part of a
viral vector, for instance a viral vector engineered
to be suitable for administration to an individual,
such as a human, and preferably additionally tumour
20   targetting.

Suitable vectors can be chosen or constructed,
containing appropriate regulatory sequences, including
promoter sequences, terminator fragments,
polyadenylation sequences, enhancer sequences, marker
25   genes and other sequences as appropriate. Vectors may
be plasmids, viral, as appropriate. For further
details see, for example, *Molecular Cloning: a
Laboratory Manual:* 2nd edition, Sambrook et al.,

1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of

5 DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al., along with all

10 other documents cited herein, are incorporated by reference.

In accordance with the present invention, compositions provided may be administered to individuals. Administration is preferably in a

15 "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature

20 and severity of what is being treated. Prescription of treatment, eg decisions on dosage etc, is within the responsibility of general practioners and other medical doctors.

A composition may be administered alone or in

25 combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the

present invention, and for use in accordance with the
present invention, may comprise, in addition to active
ingredient, a pharmaceutically acceptable excipient,
carrier, buffer, stabiliser or other materials well
5   known to those skilled in the art. Such materials
should be non-toxic and should not interfere with the
efficacy of the active ingredient. The precise nature
of the carrier or other material will depend on the
route of administration

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Experimental support for the present invention
will now be described by way of illustration. Various
additional aspects and embodiments of the present
invention will be apparent to those skilled in the
15   art. The following figures are referred to:

Figure 1 shows an exemplary embodiment of
constructs according to the present invention. The
Type I unit (1) (right-hand side) has a suppressor
binding site (2) downstream of the promoter (3); in
20   alternative constructs the suppressor binding site in
the Type I unit is upstream of the promoter. The
suppressor binding site may be absent if the Type II
unit (4) expresses antisense RNA or a ribozyme. In
the Type II promoter (5), the p53 binding site
25   (GC3p53) (6) may be upstream or downstream of the
minimal promoter element (7). The insulators (8)
indicated as being between the Type I and Type II
units may not be necessary.

Figure 2 shows schematic representations of two

constructs (Figure 2A) comprising a luciferase

encoding sequence under the control of a HSV tk

promoter (-109 to +52) and a GC3p53 sequence. In

5   GC3p53tk-luc (right-hand side of the figure), the

GC3p53 sequence is upstream of the tk promoter

element; in tkGC3p53-luc (left-hand side of the

figure) it is downstream. The results of transfection

assays are shown in Table 1: 1µg tkGC3p53-luc (lanes

10   1-4) or GC3p53tk-luc (lanes 5-8) was co-transfected

with 1µg pCI (an empty expression vector having a CMV

promoter), 0.5 and 1.5µg of the wtp53 and 1 µg mutant

p53 (mt3: $_{143}$ala-val) constructs in which the p53 gene

is under control of the CMV promoter into K562 cells.

15   Figure 2B shows the ratio of the luciferase activity

in extracts from the transfected cells with either

wtp53 or mtp53, over that with pCI (as shown in the

"ratio" column of Table 1). Figure 2C shows the

actual luciferase activity. The conclusion is that

20   the tkGC3p53 element shows higher background activity

in the absence of wild type p53 than the GC3p53tk

construct, but the latter shows much stronger relative

inducibility by p53 co-transfection.

Figure 3 shows (Figure 3A) a schematic

25   representation of constructs which comprise a full

length antisense luciferase coding sequence under the

control of the CMV promoter (construct pCMV-aluc-poly

A+) or a GC3p53-Tk promoter (construct GC3p53pt109-

aluc-poly A+), and (Figure 3B) a schematic

representation of construct GC3p53pt109-aluc(-)poly

A+, which comprises in antisense orientation part of

the luciferase coding sequence (+582 - +1) under the

5   control of the GC3p53-Tk promoter.   Table 2 shows the

results of transfection assays in the presence and

absence of wild-type and mutant p53. 1μg HSp70-luc

was co-transfected with the control vector pCI (lanes

1, 4 and 7), wtp53 (lanes 2, 5 and 8) or mt 143 (lanes

10   3, 6 and 9). GC3p53pt109-aluc-poly A+, GC3p53pt109-

aluc-(-)poly A+ and pCI-aluc were also transfected

into K562 cells (lanes 4-6, 7-9, and 10,

respectively). After a two-day culture, the

luciferase activity was measured and the ratios over

15   lane 1 (for lanes 1-3, 10), lane 4(for lanes 4-6) and

lane 7 (for lanes 7-9) were used to illustrate the

suppressing effects of the wtp53 alone and the

antisense combined. This is also illustrated in

Figure 3C in the form of a plot.

20   Figure 4 shows: Figure 4A - the sequence of a

ribozyme designed to target a 15 base pair substrate

sequence within the luciferase gene, and the substrate

sequence (from 716-731 of the luc gene of pGL3-basic);

Figure 4B - the sequences of two complementary

25   oligonucleotides (lucRa and lucRb). The reannealed

oligos were cloned into SalI/SmaI sites of the

expression vector pCI. Figure 4C - a schematic

representation of constructs pCI-luc-ribo(9) (left-

hand side of the figure), comprising the ribozyme

under the control of a CMV promoter and including an

intron sequence, and pCI-luc-ribo*(2) (right-hand side

of the figure), which is an intron-deleted derivative

5   of the former construct, by PstI/KpnI digestion

followed by self-ligation/transfection into bacteria;

Table 3 shows results of transfection assays with the

constructs: 1μg Hsp70-luc, in which the luciferase

gene is under the control of human Hsp70 promoter

10   (from -117 to +26), or was co-transfected with 1 or 2

μg pCI (lanes 1 and 4), 1 or 2 μg pCI-luc-ribo(9)

(lanes 2 and 5), and 1 or 2 μg pCI-luc-ribo*(2) (lanes

3 and 6). The luciferase activity was measured after

two days'culture. The effect of the ribozyme over the

15   expression of luciferase is presented as the ratio of

the luciferase activity of each transfectant over the

transfectants with pCI DNA (column headed "ratio").

This ratio is also shown in plot form in Figure 4D.

Background references for ribozymes include Kashani-

20   Sabet and Scanlon, 1995, *Cancer Gene Therapy*, 2(3):

213-223, and Mercola and Cohen, 1995, *Cancer Gene*

Therapy, 2(1), 47-59.

Figure 5: Figure 5A shows Type I genetic unit

constructs comprising a luciferase coding sequence

25   under the control of the HSP70 promoter. In Hsp-LacO-

luc (1), three copies of the Lac Operator sequence lie

between the promoter and the coding sequence. In Hsp-

TetO-luc (2), seven copies of the Tet Operator

22

sequence lie between the promoter and coding

sequences. Figure 5B shows Type II genetic unit

constructs comprising the GC3p53 sequence and HSV tk

promoter operationally joined to a coding sequence

5   which is in GC3p53tk-LacR (1) for the Lac Repressor

and in GC3p53tk-TetR-KRAB (2) for a fusion protein

comprising the DNA binding domain of the Tet Repressor

and the suppression domain of the KRAB repressor of

Drosophila. Table 4 shows the results of

10   cotransfection experiments using the Type I and Type

II genetic unit constructs of Figures 5A and 5B:

Hsp70-lacO-luc was transfected into K562 cells with

pCI (lane 1), p3'ss which encodes the lac repressor

under the control of a F9-1 polyoma virus promoter

15   (lane 2), wtp53 (lane 3), wtp53 + GC3p53tklacR (lane

4), mtp53 143 (lane 5) and mtp53 (143ala>val) +

GC3p53tklacR (lane 6). Hsp70-tetO7-luc was

transfected into K562 cells with pCI (lane 7), CMV-

tetR-KRAB (lane 8), wtp 53 (lane 9), wtp53 + GC3p53tk-

20   tetR-KRAB (lane 10), mtp53 143 (lane 11) and mtp53 +

GC3p53tk-tetR-KRAB (lane 12). The luciferase activity

was measured after an overnight culture and the

suppression effect presented as the ratio of the

luciferase activity of each transfectant (lanes 2-6

25   and 8-12) over that of the transfectants in lane 1 and

7, respectively. Figure 5C shows graphically the

results given in the column marked "ratio" in Table 4.

Figure 6: Figure 6A shows a Type I genetic unit

23

construct comprising the luciferase coding sequence
under the control of the Hsp70 promoter, with a tandem
repeat of the recognition site of the yeast
transcription factor GAL-4 between the coding sequence
5   and promoter. Figure 6B shows Type II genetic unit
constructs comprising an actin promoter (ref. 30)
controlling expression of a fusion protein which
comprises the GAL4 DNA binding domain and (1) the
BCDEF domain of *Drosophila* even-skipped transcription
10   factor, (2) the CDEF domain of *Drosophila* even-
skipped. Table 5 shows the results of transfection
experiments in which 1μg Hsp70-GAL4-luc was
transfected into K562 cells with 1μg pCI (lane 1),
wtp53 (lane 2), BCDEF (lane 3) and CDEF (lane 4). The
15   luciferase activity from each was measured after an
overnight culture and the suppression effect was
presented as the ratio of the luc activity lanes 2-4
over that of lane 1. Figure 6C shows this ratio in
graphical form. The Hsp70 promoter maintains its
20   original response to p53 regulation even after
insertion of the sequence containing the GAL4
recognition site. The specific suppression by the
Drosophila even-skipped protein is about 40%.

Figure 7 shows the results of experiments in
25   which a genetic unit I (pCI-GUS) comprising GUS under
the control of the CMV promoter, and a type II genetic
unit (GC3P53tk-luc) comprising luciferase under the
control of the tk promoter and the GC3p53 element were

24

transfected into cells, with or without co-
transfection of a construct expressing wild-type p53.
(Columns 1 and 3 are Luc Series 1; columns 2 and 4 are
GUS Series 2). Results are shown in Table 6.

5   Figure 8 shows the results of the TK-GCV cell
killing assay with the Hsp70-lacO-tk system in Saos-2
cells. In Figure 8A the ratio of luciferase activity
of Saos-2 cell extracts measured on the day after
transfection with (1) tk-luc or (2) GC3p53tk-luc is
10 plotted (luciferase activity of the extracts by
GC3p53tk-luc over that by tk-luc). Figure 8B shows
the ratio of the mean of absorbance at 570nm of cells
transfected with various constructs and treated with
various concentrations of GCV over the no-GCV control
15 was calculated and plotted against GCV concentration
(M) (as indicated in this figure).

    Figure 9 shows the TK-GCV cell killing assay with
the Hsp70-lacO-tk system in MCF-7 cells. In Figure 9A
the ratio of luciferase activity of MCF-7 cell
20 extracts measured on the day after transfection with
(1) tk-luc or (2) GC3p53tk-luc is plotted (luciferase
activity of the extracts by GC3p53tk-luc over that by
tk-luc). Figure 9B shows the ratio of the mean of
absorbance at 570nm of cells transfected with various
25 constructs and treated with various concentrations of
GCV over the no-GCV control was calculated and plotted
against GCV concentration (M) (as indicated in this
figure).

Figure 10 shows the TK-GCV cell killing assay with the Hsp70-lacO-tk system in MHG-U1 cells. In Figure 10A the ratio of luciferase activity of MHG-U1 cell extracts measured on the day after transfection with (1) tk-luc or (2) GC3p53tk-luc is plotted (luciferase activity of the extracts by GC3p53tk-luc over that by tk-luc). Figure 10B shows the ratio of the mean of absorbance at 570nm of cells transfected with various constructs and treated with various concentrations of GCV over the no-GCV control was calculated and plotted against GCV concentration (M) (as indicated in this figure).

Figure 11 illustrates constructs used in assays employing linked Genetic Units I and II. Figure 11A illustrates construct Hsp70-lacO-rl. Figure 11B illustrates constuct ln/tkGC3p53-luc/ln. Figure 11C illustrates construct tkGC3p53-luc. Figure 11D illustrates constructs A/B(+), A/B(-), A/C(+) and A/C(-).

Figure 12 provides information on experiments employing constructs illustrated in Figure 11, i.e. dual control constructs containing both Genetic Units I and II. Figure 12A provides a key to the constructs employed in the experiments whose results are shown in Figures 12B and 12C. The x-axis numbers in Figures 12B and 12C are given down the side of Figure 12A, indicating which combination of vectors 1-9 (across the top of Figure 12A) were employed in the

experiments whose results are shown in Figures 12B and

12C. The vectors were as follows: 1 - A/B(+):2 μg/2

μl; 2 - A/B(-):2 μg/1.97 μl; 3 - A/C(+):2 μg/2.19 μl;

4 - A/C(-):2 μg/3.6 μl; 5 - Hsp70-lac0-rl :1 μg/0.7

μl; 6 - tkGC$_3$p53-luc:1 μg/1.1 μl; 7 - pCI:1 μg/0.4 μl;

8 - pCMVwtp53:1 μg/1.1 μl; 9 - In/tkGC$_3$p53-luc/In:1

μg/0.517 μl.

Figure 12B shows the ratio of the Renilla

luciferase activity of extracts of cells co-

transfected with wtp53 over those co-transfected with

the p53- vector pCI. Figure 12C shows the ratio of

the firefly luciferase activity of extracts of the

cells co-transfected with wtp53 over those co-

transfected with pCI.

Figure 13 provides information on experiments

performed using constructs as follows: 1 - tkGC$_3$p53-

luc:1 μg/1.76 μl; 2 - In/tkGC$_3$p53-luc/In(3)+:1 μg/0.74

μl; 3 - GC$_3$p53tk-luc:1 μg/1.1 μl; 4 - In/GC$_3$p53tk-

luc/In(4)+:1 μg/0.517 μl; 5 - pCMVwtp53:1 μg/1.1 μl; 6

- pCl:1 μg/0.4 μl; 7 - Hsp70-lac0-rl(2):1 μg/0.73 μl.

Details of the combinations 1-8 of constructs 1-7 used

in the experiments for which results are given in

Figures 13B, 13C and 13D, are given in Figure 13A.

K562 cells were transfected with the combinations of

constructs and both Firefly and Renillar luciferase

activities were measured. Figure 13B shows the ratio

of the Firefly luciferase activity between each pair

of the transfection. The actual Firefly luciferase

27

activity of the corresponding extracts are shown in
Figure 13C. Figure 13D shows the down-regulation
effect of wtp53 on the Hsp70 promoter in the Hsp70-
lacO-rI system.

5   Figure 14 shows results of experiments on p53
stabilisation in tumour cells.

           Figure 14A shows a Western blot of cell lysates
with polyclonal rabbit serum that specifically detects
mouse and human p53 protein. The endogenous p53

10   protein of the MCF7 tumour cell line is unstable and
barely detectable (first lane of gel) unless the cells
are irradiated with UV light, when p53 becomes stable
(second lane). A new line of MCF7 cells was isolated
which also express a mouse p53 (from plasmid

15   Mop53His169). The endogenous human and the introduced
mouse p53 protein (which can be differentiated from
human p53 by its slightly higher mobility on the gel)
are again barely detectable (third lane) unless the
cells are irradiated with UV light (fourth lane) when

20   both proteins become stable.

           Figure 14B shows a Western blot of cell lysates
with polyclonal rabbit serum that specifically detects
human p53 protein. A new line of MCF7 cells
containing pCDNA3 Hup53-tk was isolated. Plasmid

25   pCDNA3 Hup53-tk expresses a fusion protein consisting
of full length p53 fused to the N-terminusand of full
length thymidine kinase. The endogenous normal human
p53 and the fusion protein are again barely detectable

(first lane) unless the cells are irradiated with UV
light (second lane) when both the normal p53 and the
fusion protein become stable. Regulation of stability
has therefore been conferred on the tk protein by

5　　expressing it as a fusion protein with p53 protein.

All documents mentioned in this document are
incorporated by reference.

10　*Results on transcriptional control*

Preliminary experiments

A series of constructs have been prepared and
used in transfection assays with a luciferase reporter

15　gene to test the feasibility of the proposed
approaches.

i.　Firstly, using a transient co-transfection assay
into a p53-minus human leukaemia cell line, K562, we

20　have reproduced the down-regulation effect of wtp53 on
the promoter function of four different promoters: the
SV40 early promoter, the HSV-thymidine kinase (tk)
promoter (-109 to +52) or (-81 to +52), the CMV
promoter and the human HSP70 promoter. Three mutant

25　p53 genes (143, 248 and 216) tested had lost this
suppression (down-regulation) function.

For example, using the HSP70 promoter construct,
taking as unity the level of luciferase reporter gene

29

expression obtained from 1μg of vector, a wtp53 dosage

of 0.25μg reduced the expression to 0.708, while a

dosage of 0.8μg reduced it to 0.302. On the other

hand, 1μg of a mutant p53 (mt143) increased the

5   expression level to 1.11.

ii.   Secondly, the GC3 element of the p53 recognition

sequence element (GC3p53) in the human Rb promoter

[35] was cloned upstream of the HSV-tk promoter (-109

10   to +52) or (-81 to +52), as well as the mouse fos

minimal promoter (-56 to +166). Co-transfection with

wtp53 was found to enhance the reporter gene

expression dramatically by a factor of approximately

350-, 90- and 30-fold, respectively. None of the

15   three mutant p53 genes had a detectable enhancing

effect.

The dosage effect on the transactivation function

of wtp53 was tested, using the GC3p53 construct with

the HSV-tk promoter (-109 to +52). Taking the

20   baseline level of expression as unity using 1μg vector

alone (ie zero wtp53 dosage), the relative expression

levels of the luciferase reporter with wtp53 dosages

of 0.03μg, 0.1μg, 0.3μg and 1μg were 1.75, 32, 142.7

and 393.5 respectively. In another experiment, 0.25μg

25   wtp53 increased the expression level to 40.41, and

0.8μg increased it to 104.5. On the other hand 1μg of

a mutant p53 (mt143) reduced expression to 0.54.

The GC3 element in the human Rb promoter is able

to enhance p53 mediated transactivation as shown by
Shiio et al [35]. Using constructs in which the CAT
reporter gene is expressed from the SV40 early
promoter, these authors showed that when the promoter

5   was preceded by the p53 binding site from the Rb
promoter, namely $(GGACTTGCCT)_2$, it was found to
enhance CAT expression to 22 as compared with level of
0.81 in the absence of the p53 binding site. In
another construct the p53 binding site was preceded by

10   the GC3 element, and CAT expression was enhanced to
122. On the other hand, just using a pair of GC3
elements (ie without the p53 binding site) did not
enhance expression over the control.

We tested the function of the GC3p53 response

15   element in alternative orientations, upstream of HSV-
tk promoters (-81) and (-109) respectively. Although
the levels of expression varied somewhat (for a $0.25\mu g$
wtp53 dosage), the differences did not consisently
favour one orientation over the other (for the (-81)

20   promoter it was 30 in the forward orientation and 93
in the backward orientation, while for the (-109)
promoter it was 41 in the forward orientation and 27
in the backward orientation). We therefore conclude
that this element is probably orientation independent.

25

iii. Thirdly, constructs designed to express antisense
fragments of the luciferase and thymidine kinase genes
driven by the HSVTK promoter and the GC3p53 response

element have been made and tested in transient co-transfection assays. Essentially, we used three constructs: (1) had just the HSP70 promoter expressing the luciferase reporter gene; (2) had the HSV-tk (-

5   109) promoter with the GC3p53 recognition sequence transcribing the luciferase gene in reverse orientation, followed by a polyA+ signal; (3) was similar to (2) except that it only used a 5' portion of the luciferase gene (terminating at a Bcl2 site).

10   Each of the three constructs was tested for luciferase expression, (a) with the vector alone at 1μg dosage, (b) with the addition of wtp53 at 0.25μg dosage, and (c) with the addition of mutant p53 (mt143) at 1μg dosage. The results suggested that it

15   is indeed possible to achieve down-regulation of luciferase expression using this anti-sense approach.

Further experiments

20   a.   *Transcriptional Activity of Normal and Mutant p53 Alleles*

       A series of different p53 mutants have been prepared and tested for activity with genetic units I and II using luciferase reporter genes. In addition

25   to mutants $143^{ala-val}$, $216^{val-gly}$ and $248^{arg-trp}$, previously tested, the following have been tested: $175^{arg-his}$, $245^{gly-ser}$, $248^{arg-gln}$, $249^{arg-ser}$ and $273^{arg-his}$, which together represent approximately 30% of the

total of 3870 mutations reported in the literature for

p53 in human tumours.

Extensive transfection analyses with these

mutants have been carried out and the results show

5   that the mutants are unable to stimulate transcription

through the GC3p53 consensus sequence in genetic unit

II. This provides an indication that the construct

should be fully functional in at least 30% of human

tumours carrying p53 mutations, and probably in a

10   significantly higher proportion.

b.   *Arrangement of the GC3p53 Element in Genetic Unit*

II

Since it was demonstrated that the GC3p53 element

15   can function in an orientation-independent manner, it

is possible that the arrangement wherein the element

is placed upstream from the Type II promoter (e.g. as

illustrated in Figure 1A) might not work optimally

when both units I and II are put together in one

20   molecule.

Construct GC3p53tk-luc was made with the p53

consensus binding site upstream of the minimal tk

promoter joined to a luciferase encoding sequence.

Transfection assays were performed in the presence and

25   absence of wild-type p53, as described. Results were

compared with those obtained with a further construct

(tkGC3p53-luc) in which the GC3p53 element was placed

downstream of the tk promoter and which was made in

33

order to minimise potential interference with the
functioning of genetic unit I by the GC3p53 element in
genetic element II.

The results of the transfection assays are shown
5   in Figure 2. The construct containing the GC3p53
element downstream of the minimal tk promoter
exhibited a higher background level in the absence of
wild-type p53 than the other construct. However, the
induced level in the presence of wild-type p53 (530 x
10   10^4 luciferase units) was also considerably higher.
This provides indication that more efficient
suppression of expression from genetic unit I may be
attained by having GC3p53 downstream from the tk
promoter in genetic unit II. Additional experiments
15   will determine the optimal configuration in a ligated
dual construct.

c.   *The Choice of Suppression System in Unit II*
Three possible types of suppressor unit out of
20   the various approaches available in the art have been
tested - antisense, ribozyme and transcriptional
suppressors - and each has been shown to be effective
in suppressing transcription from genetic unit I.

25   c(i) *The Antisense Approach*
Two antisense luciferase gene constructs ("Type
II genetic units") have been made and tested (see
Figure 3). These comprise either a full length

construct (A) or one containing only the 5' end of the
gene from position +582 - +1 (B). As shown in Figure
3 the luciferase gene under the control of the Hsp70
promoter ("Type I genetic unit") was suppressed by
wild type p53 down to a level corresponding to 50% of
the control (compare columns 1 and 2). Co-
transfection of the antisense constructs A or B with
the Type I construct led to a further reduction in
luciferase activity to 40% or 25% of control levels
respectively (columns 5 and 8 respectively). Further
controls showed that the full length antisense
construct driven by a CMV promoter suppressed
transcription from genetic unit I to about 37% of
control levels (column 10). Mutant p53 did not
activate transcription of the antisense constructs or
lead to suppression of luciferase activity (compare
columns 6 and 9 with 5 and 8 respectively). These
results therefore indicate that expression of
antisense luciferase constructs from genetic unit II
can suppress the activity of genetic unit I.

c(ii) *The Ribozyme Approach*

A ribozyme designed to target a 15 base pair
sequence from 731 to 716 within the luciferase gene
has been placed under the control of a CMV promoter,
with or without an intron sequence (constructs shown
in Figure 4) ("Type II genetic units"). The construct
with the intron was shown in co-transfection

experiments to reduce the level of luciferase activity
from the Hsp70 promoter ("Type I genetic unit") to 75%
(1 µg, column 2) or 68% (2 µg, column 5) of control
levels (column 1), whereas the construct without the

5   intron had very little effect. Preliminary
experiments suggest that this reduction effect may be
increased by more prolonged incubation of the cells
with the ribozyme before carrying out luciferase
assays.

10

c(iii) *The Transcriptional Suppressor Approach*
        Three widely used transcription suppressor
elements have been tested using reporter gene
constructs.

15

The lac operator suppressor
        Three copies of the lac operator sequence were
inserted downstream of the Hsp70 promoter in unit I
and tested both for responsiveness to wild type p53

20   and to co-expression of the lac repressor. Expression
of the lac repressor from a constitutive promoter
reduced luciferase activity from the Hsp70-lac 0-luc
construct to about 60% of control values (Figure 5,
compare columns 1 and 2). Co-transfection of wt p53

25   and the genetic unit 2 construct containing the lac
repressor reduced the luciferase activity to about 35%
(column 4).

The KRAB suppressor

Seven copies of the tetracycline operator
sequence were inserted downstream of the Hsp70
promoter in genetic Unit I. This promoter element
5   maintains its responsiveness to the suppressive
effects of the wild type p53 protein which causes
reduction of luciferase expression to 46% of the
control values. A construct comprising the GC3p53
element with the tk minimal promoter linked to the
10   tetracycline response element/KRAB transcriptional
suppressor has been prepared. This construct
comprises the DNA-binding domain of the tet repressor
fused with the suppression domain of the Drosophila
KRAB transcription factor. The activity of this
15   repression system was demonstrated in a co-
transfection experiment of the Hsp70 tet07 luciferase
construct together with the tet/KRAB repressor under
the control of a strong CMV promoter (Figure 5). This
reduced the luciferase activity from the Hsp promoter
20   to approximately 24% of control values (compare
columns 7 and 8). Wild type p53 suppressed the Hsp70
tet07 luciferase construct to 46% of control values
(column 9) and this was further suppressed by co-
expression of the tet/KRAB fusion protein from the
25   GC3p53 tk promoter element (to 33% - column 10).
Mutant p53 on the other hand had no effect on the Hsp
promoter with or without inclusion of the tet/KRAB
fusion construct (columns 11 and 12). These studies

clearly demonstrate the potential of KRAB to act as a
transcriptional suppressor *in trans* of the Hsp
promoter in genetic unit I.

5   *The Gal-4 suppression system*

A construct has been prepared comprising a tandem
repeat of the Gal-4 recognition sequence inserted
between the Hsp70 promoter and the luciferase reporter
gene. As shown in Figure 6, the promoter in this
10   construct maintains the property of suppression by
wild type p53. The promoter activity was also
suppressed to 40% of control values by co-transfection
of a construct comprising the Gal-4 DNA binding domain
fused with the suppression domain of the Drosophila
15   even skipped transcription factor under the control of
an actin promoter.

These results demonstrate suppression of genetic
unit I comprising the Hsp70 promoter, using various
20   different transcriptional suppressor elements.

d.   *Dual Transcriptional Control by wtp53*

We have demonstrated conclusively that within the
same transfected cells genetic units I and II behave
25   independently and predictably in response to wild-type
p53, i.e. expression from genetic unit I is suppressed
and expression from genetic unit II is up-regulated.

Co-expression of different reporter genes was

employed to investigate further the specificity of this response. In addition to luciferase (employed in the experiments described above), four reporter genes have been characterised: β-galactosidase, secreted

5   alkaline phosphatase, β-glucoronidase (GUS) (Clontech) and Renilla luciferase (RL) (Promega). An extensive series of transfection experiments has shown the best reporter gene to be GUS (Figure 7) and RL (Figures 11-13), the other two of the four giving high levels of

10   background activity.

Figure 7 shows In the absence of wild-type p53 the GUS reporter activity in K562 cells was high from the CMV promoter ($>2 \times 10^6$ units, column 2), whereas luciferase activity driven by the GC3p53tk promoter

15   was essentially at background levels (0.019×10^6 units, column 1). Expression of wild-type p53 leads to an approximately 145-fold up-regulation of luciferase activity from the GC3p53tk promoter (column 3), together with a simultaneous down-regulation (to

20   0.44 of control levels) of GUS activity from the CMV promoter (a type I promoter, column 4) (see Figure 7). This result demonstrates that genetic units I and II, when co-transfected into the same cells, respond differently to expression of wtp53. Genetic unit I is

25   suppressed, while genetic unit II is strongly stimulated by p53.

Demonstration of p53-dependent cell killing by pro-

drug activation

The experiments described above demonstrated that
reporter constructs under the control of genetic units
I and II behave in the predicted manner in the

5   presence of wild-type or mutant p53.   A further series
of constructs was prepared in which the thymidine
kinase (TK) gene was placed in genetic unit I under
the control of the HSP-70 promoter.   The recognition
sequence for the lac repressor was inserted between

10   the promoter and coding sequences.   Genetic unit II
contained the coding sequence for the lac repressor
driven by the GC3p53tk or tkGC3p53 promoter elements.

These constructs were transfected into SAOS-2
cells, reported to be p53 null.   Control transfections

15   showed that the transfection efficiency for these
cells is approximately 20% and that there is no
detectable wild type p53 activity using a GC3p53tk
luciferase reporter construct (Figure 8A).   A cell
killing assay was used to determine the effect of

20   gancyclovir treatment on SAOS-2 cells cotransfected
with various combinations of constructs.   Expression
of TK from genetic unit I leads to death of 40-60% of
cells at gancyclovir concentrations of 1.25×10^{-5}-
6.26×10^{-5}M (Figure 8B).   The observation that the level

25   of cell killing exceeds the level expected from this
efficiency of transfection demonstrates that some of
the cells are being killed, as expected due to a
bystander effect.   Inclusion of the GC3p53tk-lacR or

tkGC3p53-lacR constructs did not appreciably affect this result, but in the presence of wt p53, in particular the tkGC3p53-lacR construct was able to reduce the level of cell killing to about 10-20%.

5 Gancyclovir by itself had no effect on cell viability at these concentrations. This result demonstrates that in the presence of wt p53m considerable suppression of the effect of genetic unit I can be achieved through upregulation of the lac

10 transcriptional repressor by the GC3p53 element.

Further tests of these constructs were carried out using cells which express endogenous normal (MCF-7 cells) or mutant (MHG-U1) p53 protein. The transfection efficiency for MCF-7 cells was higher

15 than that observed for SAOS-2 cells, and these cells clearly had wild type p53 activity (Figure 9A). Treatment of MCF-7 cells cotransfected with genetic units I and II did not lead to any observable cell death at a concentration of 6.25×10^{-5}M gancyclovir

20 (Figure 9B). This indicates that the HSP-70 promoter in genetic unit I is strongly downregulated by the wild-type endogenous p53 in MCF-7 cells. At this concentration of gancyclovir, whereas SAOS-2 cells with no wt p53 are killed to a level of at least 50%

25 by genetic unit I (against a background transfection efficiency in this transient assay of 20%). MCF-7 cells with functional wt p53 are unaffected, even although the transfection efficiency is more than

twice as high.

Further experiments were carried out with MHG-U1 cells. Although the transfection efficiency of these cells is only 1-3%, more than 25% of the cells were killed by genetic unit 1 at a concentration of 6.25×10^{-5}M gancyclovir (Figure 10B). As expected, no effect was seen after inclusion of genetic unit 2, which is not upregulated by the mutant p53 in these cells (Figure 10A).

The experiments and results will now be described in more detail.

Cell killing assays

Cell killing assay in Saos-2 cells

Saos-2 cells (0.5-1.5 $\times 10^6$ cells in 0.3 ml) were transfected with 1 μg pSV-β-gal by electroporation (320 volts/1050 μC). After an overnight incubation, the cells were fixed and stained for the expression of the β-gal [Gossler, 1994]. 400 cells were counted and the percentage of blue cells was determined and used as a measure of transfection efficiency. By this measurement, the transfection efficiency of Saos-2 cells is approximately 20%.

Saos-2 cells were transfected with 1 μg of tk-luc or GC3p53tk-luc and the luciferase activity of the cell extracts was measured on the next day. The ratio of the luciferase activity of the extracts by GC3p53tk-luc over that by tk-luc was calculated and

plotted. As shown in Figure 8A, there is no
significant difference between the cell extracts
transfected by either tk-luc or GC3p53tk-luc. Since
p53 protein was not detectable by immunoblotting

5   analysis with p53 antibodies (DO-1 or CMV-1), this
result confirms that the p53 status of Saos-2 cells is
p53-/-.

Saos-2 cells (3 x 10^6 cells/0.3 ml) were
transfected with 1µg of Hsp70-lacO-tk along with 1µg

10 p3'SS encoding the lacR under the control of the FG-1
polyoma viral promoter, GC3p53tk-lacR or tkGC3p53-
lacR. Either pC1 (a CMV promoter vector control) or
pCMV-wtp53 (the wtp53 gene under the control of a CMV
promoter) was also included in this analysis. The MTT

15 dye-conversion assay (Promega, [Promega, 1995]) was
carried out and the ratio of the mean of absorbance at
570nm of the cells treated with various concentrations
of GCV over the no-GCV control was calculated and
plotted against GCV concentration (as indicated in

20 Figure 8B).

The GCV tolerance profile of Saos-2 cells was
determined in parallel by treating the cells with
various concentrations of GCV (ranging from 0.1 µmole
to 2 mM).

25

Cell killing assay with MCF-7 cells

MCF-7 cells (0.5-1.5 x 1^{-6} cells in 0.3 ml) were
transfected with 1 µg pSV-β-gal by electroporation

43

(320 volts/1050 μC). After an overnight incubation, the cells were fixed and stained for the expression of the β-gal [Gossler, 1994]. 400 cells were counted and the percentage of blue cells was determined and used

5 as a measure of transfection efficiency. By this measurement, the transfection efficiency of MCF-7 cells is approximately 48%.

MCF-7 cells were transfected with 1 μg of tk-luc and GC$_3$p53tk-luc, respectively, and the luciferase

10 activity of the cell extracts was measured on the next day. The ratio of the luciferase activity of the extracts by GC$_3$p53tk-luc over that by tk-luc was calculated and plotted. As shown in Figure 9A, there is 19 fold increase in luciferase activity in the

15 extract transfected by GC$_3$p53tk-luc in comparison to that by tk-luc, indicating that p53 status of MCF-7 cells is wildtype.

MCF-7 cells (3 x 10^5 cells/0.3 ml) were transfected with 1 μg of Hsp70-lacO-tk along with 1 μg

20 p3'SS, GC$_3$p53tk-lacR, or tkGC$_3$p53-lacR. The MTT dye-conversion assay (Promega, [Promega, 1995]) was carried out and the ratio of the mean of absorbance at 570$_{nm}$ of the cells treated with various concentration of GCV over the no GCV control was calculated and

25 plotted against GCV concentration (as indicated in Figure 9B).

The GCV tolerance profile of MCF-7 cells was determined in parallel by treating the cells with

44

various concentrations of GCV (ranging from 0.1 μmole

to 2 mM).

Cell killing assay with MHG-U1 cells

5   MHG-U1 cells (0.5-1.5 x 10^6 cells in 0.4 ml) were

transfected with 1 μg pSV-β-gal by electroporation

(290 volts/1050 μC). After an overnight incubation,

the cells were fixed and stained for the expression of

the β-gal [Gossler, 1994]. 400 cells were counted and

10   the percentage of the blue cells was determined and

used as a measure of transfection efficiency. By this

measurement, the transfection efficiency of MHG-U1

cells is approximately 3%.

        MHG-U1 cells were transfected with 1 μg of tk-luc

15   or GC$_3$p53tk-luc, and the luciferase activity of the

cell extracts was measured on the next day. The ratio

of the luciferase activity of the extracts by

GC$_3$p53tk-luc over that by tk-luc was calculated and

plotted. As shown in Figure 10A, there is no

20   significant difference between the cell extracts

transfected by either tk-luc or GC$_3$p53tk-luc. Since

the p53 protein was detected by immunoblotting

analysis with p53 antibodies (DO-1 or CM-1), this

result indicates that the p53 status of MGH-U1 cells

25   is mutant type.

        MGH-U1 cells (3 x 10^6 cells/0.3 ml) were

transfected with 1 μg of Hsp70-lacO-tk along with 1 μg

p3'SS, GC$_3$p53tk-lacR, or tkGC$_3$p53-lacR, respectively.

The dye-conversation assay (Promega, [Promega, 1995])
was carried out and the ratio of the mean of
absorbance at 570_{nm} of the cells treated with various
concentrations of GCV over the no GCV control was

5   calculated and plotted against GCV concentration (as
indicated in Figure 10B).

The GCV tolerance profile of MGH-U1 cells was
determined in parallel by treating the cells with
various concentrations of GCV (ranging from 0.1 μmole

10   to 2mM).

Materials and methods for cell killing assays
Cell culture and DNA transfection:

Cell lines: Saos-2 (p53-/-, human osteogenic

15   sarcoma cell line, ATCC HTB-85), MCF-7 (p53+/+, a
human mammary adenocarcinoma), and MHG-U1 (EJ cells,
human bladder carcinoma cell line, [1977] mtp53).
Cells were cultured in the required media + 10% calf
fetal serum, to their confluence. Cells were

20   recovered by trypsin digestion and resuspended in a
single cell suspension at a concentration of 5 x 10^6
to 1.5 x 10^7 cells per ml. The optimal condition for
the DNA transfection by electroporation for each cell
line has been identified by a series of piloting

25   experiments varying in voltage and volume with the
pGL3 control (Promega, at which the Firefly luciferase
gene is under the control of the SV-40 promoter and
enhancer). The electroporation condition by which the

46

cell extract has the highest luciferase activity is further confirmed by the β-gal staining [Gossler, 1994] of the transfected cells with the pSV-β-gal (Promega). The transfection efficiency of each cell line is presented as the percentage of the blue cells among 400 individual cells counted. The represented field was photographed with positive film and scanned and processed in computer.

Constructs:

A, Hsp70-lacO-tk

The human heat-shock gene promoter (-117 to +26) [Tsutsmi-Ishii, 1995] is amplified from the genomic DNA of the human HL-60 cells with *pfu* polymerase (Stratagen) and cloned at Srf I site of pCR-script™ SK(+)(Cam) (Stratagen) to create Hsp70 in sk. The 467 bp Hind III fragment from pOP13CAT (Stratagen) where three Lac operator sequences are located at an intron was cloned at Hind III site of Hsp70 in sk to create Hsp70-lacO in sk.

The coding region of the HSV thymidine kinase gene is amplified with *pfu* polymerase from pTK0 (gift from Hein) and cloned at pSI expression vector (Promega) to create pSItk. The 1.5 kb BgI II/Bam HI fragment containing the tk gene as well as polyA+ additional signal is cloned at Sal I site of Hsp70-lacO in sk to create Hsp70-lacO-tk.

B, GC₃p53tk-lacR and tkGC₃p53-lacR

47

The 180 bp Sac I/BgI II fragment containing HSV tk promoter (-109 to +52) of pT-109 [Nordeen, 1988] is cloned at the Bam HI site of pCR-script™ SK(+)(Cam) (Stratagen) to create tk-sk. GC₃p53 element from the human Rb gene promoter [Shiio, 1993] is cloned at Eco RV site of tk-sk to create tkGC₃p53-sk. GC₃p53 element is cloned at the Sma I site of pT-109 [Nordeen, 1988] to create GC₃p53pT-109. And the Sal I/BgI II fragment of GC₃p53pT-109 is cloned at Xho I/BgI II sites of pCR-script™ SK(+)(Cam) (Stratagen) to create GC₃p53tk-sk.

The 1667 bp Xba I/Hind III fragment of p3'SS (Stratagen) containing the lac I gene was cloned at the Eco RV and Hind III sites of the GC₃p53tk-sk to create GC₃p53tk-lacR.

The 1.7 kb Eco RI/Sal I fragment of GC₃p53tk-lacR is cloned at the Hind III/Sal I sites of tkGC₃p53-sk to create tkGC₃p53-lacR.

Determination of the function status of the p53 in cell

Cells were transfected with either tk-luc {(at which the Sac I/BgI II fragment containing HSV tk promoter (-109 to +52) [Nordeen, 1988] was cloned at Sac I/Hind III sites of pGL3basic (Promega)}; or GC₃p53tk-luc {(at which the GC₃p53 element is cloned at the upstream of the tk promoter of tk-luc)}. The luciferase activity of the extract of the cells that

had been transfected by GC₃p53tk-luc was compared with
that by tk-luc. A significant enhancement of the
luciferase activity by GC₃p53tk-luc over tk-luc is
used as a criterion for the wtp53 status. Otherwise,

5   the p53 status of the cell line is either p53-/- or
mtp53 that would be distinguished by the immunblotting
with p53 specific antibodies (DO-1, from D. Lane).

TK-GCV assay

10   1 μg Hsp70-lacO-tk, with 1 μg of one of the
following constructs: p3'SS (Stratagen), GC₃p53tk-
lacR, and tkGC₃p53-lacR) was transfected into 3×10^6
either MCF-7 or MGH-U1 cells by electroporation. For
Saos2-cells, one of the additional two constructs: 1,

15   pCI (Promega, an eukaryotic expression vector having
CMV promoter) and 2, pCMVwtp53 (a similar construct as
pCI in which the wtp53 is under the control of the CMV
promoter, from B. Volgestein) were also included for
transfection. After an overnight culture, the

20   transfected cells were harvested by trypsin digestion
and made up a single cell suspension at a
concentration of 10^5 cells/ml. Distribute 10^4
cells/100 μl suspension into each well (totally 24) in
the 96 well culture plate. Following a six hour

25   culture, 100 μl medium without Ganciclovir (GCV)., and
with 0.2, 1, 5, 25 or 125 μmole GCV was added into the
corresponding well, respectively. Each assay was
carried out quadruplicately. After a 3-4 day culture,

the GCV effect over the cell growth was determined by a MTT dye conversion assay (Promega, CellTitre 96™ Non-radioactive cell proliferation assay, [Promega, 1995]). In brief, 15 μl dye mixture was added into

5   each well and cells were further cultured for four hours at 37°C. Then to each well, 100 μl solubilisation solution was added. After an overnight incubation at room temperature, the absorbance of each well at 570$_{nm}$ wavelength was recorded by using an ELISA

10   reader (Emas, Molecular Device) and the mean value of the absorbance from the four identical wells were calculated. The GCV effect on the cell growth was presented by the plot of the ratio of the mean absorbance of the cell treated with various

15. concentration of GCV over that of the one without GCV against the GCV concentration.

The toxicity of the GCV to cells were analysed by subjecting the cells to various concentration of GCV (ranging from 0.1 μmole to 2 mM GCV at 37°C) for 3

20   days. The remaining steps of the treatment were carried out as the tk-GCV assay.

e.   *Linkage of Genetic Units I and II*

Genetic units I and II such as employed in "d"

25   above, i.e. respectively comprising reporter genes such as GUS and luciferase are linked on a single vector construct and transfected into cells, where the tests performed in "d" above are repeated. The units

are placed "head-to-head", "head-to-tail" or "tail-to-tail" and the spacing may be varied. Interruptor sequences may be employed between the two units.

The most successful vector construct arrangement,
5   that is the arrangement found to give the best down-regulation of (e.g.) GUS activity and up-regulation of luciferase activity in the presence of wild-type p53, is tested, with the replacement of luciferase with a repressor such as the KRAB/tet fusion repressor or
10 LacI repressor and the replacement of reporter (e.g. GUS) with tk, for tumour specific cell killing in vitro, and in vivo in nude mouse tumours.

A series of control experiments using a number of different reporter genes showed that the best
15 combination of reporters for expression in the same transfection assays was Firefly luciferase (luc) and Renilla luciferase (rl).

The most simple test of the dual control unit was to link genetic unit I, containing the HSP 70 promoter
20 with the lac operator driving the rl reporter, directly with genetic unit II comprising the tkGC3p53 promoter controlling luc expression (constructs A/C(+) and A/C(-), Figure 11). Genetic unit I functioned normally in this dual construct, and showed increased
25 luciferase activity in response to co-expression of wt p53 (Figure 12C: compare columns 5 and 7 with 6 and 8 respectively). On the other hand, the Renilla luciferase activity from the HSP promoter in genetic

unit II was also stimulated (Figure 12B: compare columns 5 and 7 with 6 and 8 respectively). This indicated that the p53 consensus sequence in genetic unit II was having a strong enhancing effect on both

5   promoters in the dual construct, and that insulator or interruptor sequences would be required.

Insulator sequences from the chick β-globin locus [42] were inserted at both ends of genetic unit II (either as tkGC3p53-luc or GC3p53tk-luc) (see Figure

10   11), and these were tested in co-transfection assays with wt p53, with or without genetic unit I driving the rl reporter gene. The results are shown in Figure 13.

The presence of the insulator sequences in unit

15   II had the effect of inhibiting the tk minimal promoter both in the absence and in the presence of wt p53. However this effect was stronger in the absence of p53, with the consequence that the degree of stimulation of luciferase activity by p53 was greater

20   than in the control constructs without the insulators (Figures 13B and 13C). Figure 13D shows results which demonstrate that rl activity from genetic unit I was suppressed in the usual way by wt p53, whether or not the insulator constructs were included in the

25   transfections.

The unit II containing the flanking insulator sequences was then directly ligated to genetic unit I driving the rl reporter, either in a head to head

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(construct A/B(+), Figure 11), or in a head to tail (construct A/B(-), Figure 11) orientation. These constructs were co-transfected into K562 cells in the presence or absence of wt p53, and the activities of

5 both luciferase reporters were measured. The results in Figure 12C show that genetic unit II within the dual control constructs is strongly stimulated by wt p53 (compare columns 1 and 3 with 2 and 4 respectively, Figure 12C). In this experiment, no

10 stimulation could be seen of the HSP-70 promoter in genetic unit I, suggesting that the activity of the p53 enhancer sequence in unit II had been successfully isolated by the insulator sequences.

The experimental work will now be described in

15 more detail.

Assays with Ligated vector constructs - dual control units

20 *Materials and methods*

Constructs and assay for linked Genetic Units I and II

Basic constructs:

Figure 11 A, Hsp70-lacO-tk and Hsp70-lacO-rl

The human heat-shock gene promoter (-117 to +26)

25 [Tsutsmi-Ishii, 1995] was amplified from the genomic DNA of the human HL-60 cells with *pfu* polymerase (Stratagen) and cloned at Srf I site of pCR-script™ SK(+)(Cam)(Stratagen) to create Hsp70 in sk. The 467

bp Hind III fragment from pOP13CAT (Stratagen) where
three Lac operator sequences are located at an intron
was cloned at Hind III site of Hsp70 in sk to create
Hsp70-lacO in sk. .

5   The coding region of the HSV thymidine kinase
gene was amplified with *pfu* polymerase from pTK0 and
cloned at pSI expression vector (Promega) to create
pSItk. The 1.5 kb BgI II/BamHI fragment containing
the tk gene as well as polyA+ additional signal was
10   cloned at SaI I site of Hsp70-lacO in sk to create
Hsp70-lacO-tk.

       The 1.2 kb Nhe I/Bam HI fragment from pRL-nuII
(Promega) containing *Renilla* luciferase gene as well
as poly A+ signal was cloned at the Cla I site of
15   Hsp70-lacO-tk (the tk gene is detected out by Cla I
digestion from Hsp70-lacO-tk) to create Hsp70-lacO-rl.
Figure 11 B, tkGC₃p53-luc

       The 180 bp Sac I/BgI II fragment containing HSV
tk promoter (-109 to +52) was cloned at the Bam HI
20   site of pCR-script™ SK(+)(Cam)(Stratagen) to create
tk-sk. GC₃p53 element from the human Rb gene promoter
[Shiio, 1993] was cloned at Eco RV site of tk-sk to
create tkGC₃p53-sk. The 2.0 kb Hind III/Sal I
fragment containing the Firefly luciferase gene and
25   poly A+ signal of pGL3Basic was cloned into tkGC₃p53-
sk to create tkGC₃p53-luc.
Figure 11 C, In/tkGC₃p53-luc/In

       The construct (pJC5-4) [Chung, 1993] containing

two insulator element from the chicken β-globin locus
as well as the local control region and neo gene was
provided by G. Felsenfeld. Deletion of both the local
control region and neo gene creates pJC5-4-2. The 2.2

5   kb Xho I/Sal I fragment of tkGC$_3$p53-luc containing the
luciferase gene under the control of both GC$_3$p53tk
elements was cloned at the Bam HI site of the pJC5-4-2
to create In/tkGC$_3$p53-luc/In.

Figure 11 D, Dual Constructs

10   .   See Figure 11D for the details of constructs
consisting of Hsp70-lacO-rl (=A) and In/tkGC$_3$p53-
luc/In (=B) or tkGC$_3$p53-luc (=C). The 2 kb BssH II
fragment containing the *Renilla* luciferase gene under
the control of Hsp70 promoter and lac Operator of

15   Hsp70-lacO-rl (=A) was cloned at Sal I site of
In/tkGC$_3$p53-luc/In (=C) to create A/B+ (referring the
head to head arrangement of the two units) and A/B-
(referring the head to tail arrangement of the two
units). The 2.2 kb Xho I/Sal I fragment of tkGC$_3$p53-

20   luc (=C) was cloned at Bst XI site of Hsp70-lacO-rl
(=A) to create A/C+ (referring to the head to tail
arrangement of the two units) and A/C- (referring to
the head to tail arrangement of the two units).

25   *Results*

     The differential effects of the wtp53 over the
Unit I and Unit II were maintained in the ligated dual
control system when the insulator sequence was

included, as shown in Figure 12. The effect of the
insulators on the Unit II response to wtp53 is shown
in Figure 13.

In the experiments of which the results are shown
5   in Figure 12, K562 cells (0.5 to 1.5 x 10^6 cells/0.4
ml) were transfected by electroporation (260 volts,
$1050\mu C$) with the constructs listed in the brief
description given above for Figure 12 and as indicated
in Figure 12A. Both Firefly and Renilla luciferase
10   activities were measured and the ratio of the
luciferase activity of the extracts being co-
transfected with wtp53 over that with pCI (p53-) was
calculated and plotted. Figure 12B shows the results
for Renilla luciferase, its gene being under the
15   control of the Hsp70 promoter, while Figure 12C shows
the results for Firefly luciferase, its gene being
under the control of HSVtk promoter and GC3p53
element.

In the experiments for which the results are
20   shown in Figure 13, K562 cells were transfected by the
constructs listed in the brief description of the
figure given above in the combinations given in Figure
13A. Both Firefly and Renilla luciferase activities
were measures in the cell extract on the next day.
25   The results shown in Figures 13B, 13C and 13D show
that insulator sequence has some inhibitory effect on
the HSVtk promoter in Genetic Unit II. Since this
effect is more potent when the p53 protein is absent

56

than when present, the insulator elements would
enlarge the difference of the potency of the Unit II
between the situations where the wtp53 is present or
absent.

5

Results on p53 mediated protein stability

One of the earliest responses to DNA damage in
normal cells is the increased expression level of p53
protein. This has been ascribed to a combination of
10   elevated protein stability, possibly due to
phosphorylation induced by a DNA damage recognition
process [36, 37], or to increased translatability of
the p53 mRNA [7]. For whatever reason, the end result
is that for a given level of mRNA, p53 protein is
15   produced and stabilised at higher levels in tumour
cells than in the corresponding normal cells [8].

In a parallel series of experiments to those
described above to exploit p53 transcriptional
control, the differential stability of p53 in normal
20   and tumour cells can be used to stabilise the TK
protein in a tumour cell environment. Although the
exact mechanism by which increased p53 protein levels
are obtained is not yet clear, we have prepared a
series of constructs designed to test stabilisation of
25   fusion proteins comprising the TK protein together
with all or various domains of wild-type p53 in the
same way as p53 itself in different tumour cell lines.

Several constructs have been prepared and shown

57

to express the appropriate fusion proteins in vitro.
These constructs can be transfected into cell lines
which exhibit different levels of expression of p53,
presumably as a consequence of differing levels of DNA

5   damage signals which they produce [8]. Modifed TK
gene fusion constructs that show similar differences
in levels of expression in the cell lines as wild-type
p53, can be used in order to ensure maximal
discrimination in overall levels of TK expression

10   between normal and tumour cells.

The cell line MCF-7 has been previously shown to
express wt p53 which is inducible by DNA damage, for
example by UV irradiation (8). MCF-7 cells were
transfected with a fusion construct encoding TK fused

15   to full length wt p53 and stable transfectants were
selected with G418. Western blotting using anti-p53
antibodies showed the presence of a fusion protein of
the anticipated molecular size in the transfectants
(Figure 14). Moreover, when these cells were

20   subjected to irradiation with UV light, the p53-TK
fusion protein was stabilised and expressed at an
increased steady-state level in a manner similar to
the endogenous p53 protein (Figure 14).

The indication is that fusion constructs of this

25   type will be stabilised and expressed at higher levels
in tumour cells expressing mutant p53 than in the
equivalent normal cells, and may be used for tumour-
specific cell killing, either in the context of the

tumour-specific gene expression constructs employing

transcriptional control as discussed, or not. Such

constructs may also be used for killing of tumour

cells which express elevated levels of wt p53, since

5   the fusion protein may be stabilised in the same way

as the endogenous p53 protein. In some tumours, wild-

type p53 is expressed but its normal function is

disrupted by interaction with other proteins, as

discussed, or by being incorrectly localised in the

10   cytoplasm - where it is stabilised. Examples include

some tumours in breast cancer (Moll et al. PNAS USA

89:7262-7266, 1992) and neuroblastoma (Moll et al.

PNAS USA 92: 4407-4411, 1995). The evidence that P53

fusion molecules are stabilised in the manner of wtP53

15   points to their use in targetting expression of a gene

product of interest in such tumour cells, i.e.

providing discriminatory expression in such tumour

cells as opposed to non-tumour cells.

20   Testing of constructs for activity in vitro and in
        vivo

        The approaches outlined above enable optimal

constructs to be arrived at for the realisation of

high expression levels in tumour versus normal cells.

25   The efficacy of these TK constructs in killing tumour

cells may be tested in a range of human and mouse cell

lines which have been characterised with respect to

their tumorigenicity and p53 status. These studies

can be carried out both on cells *in vitro* and in nude mouse tumours.

Those constructs which show the highest ability to kill tumour cells *in vitro* or *in vivo*, with minimal effects on primary or immortalised normal cells, may be injected into fertilised mouse eggs in order to generate transgenic mice. Adult animals carrying these constructs in the germ line should express very low or no TK mRNA or protein in normal tissues, but elevated levels should be detectable in tumours induced in the transgenic mice by a variety of procedures. The ability of the constructs to be switched on in a tumour cell environment will be tested by inducing skin tumours in these mice by sequential treatment with initiators and promoters of carcinogenesis. The animals will also be crossed with other strains of transgenic mice which develop tumours as a consequence of carrying an activated oncogene in the germline [38] or with animals carrying non-functional p53 or Rb genes and which spontaneously develop a variety of tumour types including lymphomas and sarcomas at high frequency. The animals will be injected with Ganciclovir in an attempt to induce tumour regression. It should be noted that because of the strategy we have adopted, the highest levels of expression and stability of TK protein would be expected in the most highly malignant tumours.

60

Further considerations

Various genes are known to incorporate elements
which confer stability or susceptibility to
degradation, either at the RNA or the protein level,
5   and these may be used to enhance stability or
degradation in our constructs, according to whichever
characteristic is required.

As regards the antitumour gene to be expressed,
HSVTK has been particularly mentioned above, as it is
10   known to convert the prodrug Ganciclovir to a
cytotoxic counterpart. However, many other
possibilities exist, or will be found, which may be
utilised in our constructs, including alternative pro-
drug activating enzymes, or cytokines such as IL-2 or
15   GM-CSF which increases immune recognition of tumours.

A virus-related approach seems particularly
interesting in connection with the present invention.
Tumour metastases occur in widely different parts of
the body, and a treatment based on systemic infection
20   by a viral vector has the potential to reach virtually
all parts of the body. Viral vectors which are used
for therapeutic purposes are normally replication-
incompetent, but we propose the use of replication-
competent viral vectors [39], which can infect all
25   cells but are capable of replication only in tumour
cells.

We envisage the use of the dual control element to
direct expression of genes which control virus

replication to tumour cells. For example a viral
polymerase gene (retrovirus or adenovirus) could be
placed under the control of the dual promoter, or the
function of viral promoters driving expression of

5   proteins required for replication could be disrupted
in normal cells by insertion of the binding site for a
repressor element controlled by wild type p53, as in
Figure 1. Tumour cell killing will be achieved using
the lytic properties of the virus (for adenovirus) or

10   by incorporation of an HSV-TK construct (or another
construct encoding a pro-drug activation enzyme) and
gancyclovir treatment. Discrimination between normal
and tumour cells may be enhanced by the induction of
wt p53 which takes place in normal cells upon

15   infection with a DNA tumour virus.

It is also known that very low dose radiation can
induce wtp53 in normal cells. Therefore such
radiation could be used locally or generally to
enhance the suppression of the anti-tumour gene (or

20   viral replication) in normal cells.

TABLE 1

Transfection assays - see Figure 2 and discussion

	tkGC3p53-luc	GC3p53tk-luc	pc1	wtp53	wtp53	mt3	Ratio*	Luc(x10⁻⁴)
1	1 μg		1 μg				1	59
2	1 μg			0.5 μg			5.79	342
3	1 μg				1.5 μg		8.98	530
4	1 μg					1 μg	0.69	40
5		1 μg	1 μg				1	1.8
7		1 μg		0.5 μg			50.33	94
8		1 μg			1.5 μg		120.6	225
9		1 μg				1 μg	0.816	1.5

TABLE 1

TABLE 2

· <u>Transfection assays - see Figure 3 and discussion</u>

GC₃p53pt109-aluc(-)poly A+				
				Ratio/ vec.
1.	Hsp 70-luc	vector	1 μg/0.52 μl		.1
2.	Hsp 70-luc	wt	0.25 μg/0.4 μl		0.57
3.	Hsp 70-luc	mt143	1 μg/0.56 μl		1.1
4.	Hsp 70-luc	vector	1 μg/0.52 μl	GC₃p53pt109-aluc-poly A+(1 μg)	1
5.	Hsp 70-luc	wt	0.25 μg/0.4 μl	GC₃p53pt109-aluc-poly A+(1 μg)	0.48
6.	Hsp 70-luc	mt143	1 μg/0.56 μl	GC₃p53pt109-aluc-poly A+(1 μg)	1.15
7.	Hsp 70-luc	vector	1 μg/0.52 μl	GC₃p53pt109-aluc(-)poly A+(1 μg)	1
8.	Hsp 70-luc	wt	0.25 μg/0.4 μl	GC₃p53pt109-aluc(-)poly A+(1 μg)	0.75
9.	Hsp 70-luc	mt143	1 μg/0.56 μl	GC₃p53pt109-aluc(-)poly A+(1 μg)	1.05
10.	Hsp 70-luc			pCl-aluc-polyA+(1 μg)	0.36

64

TABLE 3

Transfection assays - see Figure 4 and discussion

	Hsp70-tet07-luc	pC1	pC1-luc-ribo (9)	pC1-luc-ribo*(2)	Ratio
1	1.2 μl	0.48 μl			1
2	1.2 μl		0.286 μl		0.75
3	1.2 μl			0.3 μl	0.95
4	1.2 μl	0.96 μl			1
5	1.2 μl		0.572 μl		0.68
6	1.2 μl			0.6 μl	1.02

TABLE 4

<u>Transfection assays - see Figure 5 and discussion</u>

Hsp70-lacO-luc		pCl	p3'SS*	wtp53	GC$_3$p53tk-lacR	mtp53**	Ratio
1	1 µg	1 µg					1
2	1 µg		1 µg				0.623
3	1 µg			0.5 µg			0.43
4	1 µg			0.5 µg	1 µg		0.35
5	1 µg					1 µg	1.1
6	1 µg				1 µg	1 µg	0.98
Hsp70-tet07-luc		(CMV-tetR-KRAB)			GC$_3$p53tk-tetR-KRAB)		
7	1 µg	1 µg					1
8	1 µg		0.28 µl				0.24
9	1 µg			0.5 µl			0.46
10	1 µg			0.5 µl	0.62 µl		0.33
11	1 µg					0.56 µl	0.95
12	1 µg				0.62 µl	0.56 µl	0.99

66

TABLE 5

<u>Transfection assays - see Figure 6 and discussion</u>

	Hsp70-GA14-luc(8)	pC1	wtp53	BCDEF	CDEF	Ratio
1	1 μg	1 μg				1
2	1 μg		1 μg			0.63
3	1 μg			1 μg		0.8
4	1 μg				1 μg	0.56

TABLE 6

<u>Transfection assays - see Figure 7 and discussion</u>

	A	B	C	D	E	F	G	H	I
1		pC1-GUS	GC$_3$p53tk-luc	pC1	wtp53	luc		GUS	
2	1	1 μg	1 μg	1 μg		19280	1	2092000	1
3	2	1 μg	1 μg		1 μg	1926600	145	914960	0.44

G: Ratio of the luciferase activity of the extract co-transfected with wtp53 construct over that with pC1.

I: Ratio of the GUS activity of the extract co-transfected with wtp53 construct over that with pC1.

68

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69

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